A POLAROGRAPHIC INVESTIGATION OF AMINO ACIDS

By
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INTRODUCTION

Since the polarographic method was developed by Heyrovsky (1) in 1922, a large amount of work has been done on qualitative and quantitative identification of electro-reducible or electro-oxidizable substances with the polarograph. The instrument has also been used to study the structures of compounds, reaction mechanisms, and reaction rates.

The general principles of polarography are presented in a two-volume monograph by Kolthoff and Lingane (2). They discuss theoretical principles, instrumentation, and methods in Volume I and some practical applications in Volume II.

The experimental work reported in this dissertation was undertaken in order to study several amino acids polarographically. Half-wave potentials were determined on the amino acids studied that were found to produce polarographic waves. In addition, the effect of concentration of amino acid on the wave height is reported and a possible mechanism of the reduction is proposed.

The first polarographic work involving amino acids was stimulated by the discovery by Herles and Vancura (3) that aqueous solutions of serum from human blood exhibited a polarographic wave which began at -1.6 volts. Since the wave appeared at voltages just before the wave of the sodium ion, it was termed the "prenatrium wave." Heyrovsky and Babicka (4) continued this investigation and attributed the wave to the

presence of both albumin and ammonium ions, since no wave appeared in the absence of either one and the wave heights were found to be proportional to the concentration of both. They found that either glycine or asparagine could be substituted for ammonium ions to produce this albumin wave. Since albumins consist chiefly of long polypeptide chains of amino acids, they tried adding amino acids to solutions of ammonium salts, but found that no wave was produced. The presence of undecomposed albumin with either ammonium ions or an amino acid was necessary for the appearance of the wave.

The theory of the prenatrium wave was based on the mechanism of the reduction of ammonium ions which, in the absence of albumin, produce a wave beginning at -1.7 volts. The mechanism they used was:

$$2\,e + 2\,NH_4^+ \longrightarrow 2\,NH_3 + H_2 .$$

Adsorption of ammonium ions (or amino acid) on the albumin molecules loosens the N-H bond and consequently reduction occurs more easily, i.e., at a more positive potential.

Bridicka (5) discovered another polarographic effect of proteins which led to further investigation of amino acids. While electrolyzing hexammine cobaltic chloride in ammonia-ammonium chloride buffer at the dropping mercury electrode, he added small amounts of blood serum to suppress the cobalt maximum. Two characteristic waves, referred to as a double wave, were observed which did not coincide with the prenatrium wave found by Herles and Vancura. Extracts of coagulated proteins gave qualitatively the same waves as uncoagulated proteins indicating that some aggregate smaller than the protein molecule itself could produce

the same effect. To determine the nature of the protein constituent which caused the observed phenomenon, he investigated individual amino acids in buffered cobalt solutions (6). No characteristic wave was produced when glycine, asparagine, arginine, leucine, or tyrosine was substituted for the undecomposed protein. However, when cystine or cysteine was substituted a wave was produced. Since the wave produced by cystine in buffered cobalt solutions was 500 times as large as would be expected from the reduction of cystine only, he explained the effect as a catalysis of the deposition of hydrogen. In the absence of cobalt salts, no "catalytic" wave was observed, but a wave due to the reduction of cystine was produced; thus, cystine was the first amino acid to be reduced polarographically. Application of the polarographic method for the determination of per cent cystine in protein hydrolyzates was feasible and Bridicka used this method to analyze human hair, wool, egg albumin, and other proteins for cystine.

The following mechanism was proposed by Bridicka (7) to explain the catalytic wave: Cysteine forms a strong complex with cobaltous ions. This loosens the S-H bond, allowing the electrodeposition of hydrogen according to the following reaction:

$$2\ RS\text{-}H + 2\ e \longrightarrow 2\ RS^- + H_2 .$$

The RS^-, being a base, then reacts with the buffer to restore the sulfhydryl group.

$$RS^- + HB \longrightarrow RS\text{-}H + B^- .$$

Sladek and Lipschutz (8) found that hydrolyzates of proteins from liver had a powerful suppressive effect on the wave produced by

cysteine (or cystine) in buffered cobalt solutions. Individual amino acids were studied to determine their effect on the catalytic cysteine wave. Glycine, α- or β-alanine, tyrosine, or lysine had no effect. Arginine, β-phenyl- α-alanine, β-phenyl- β-alanine, tryptophan, or histidine was found to suppress the cysteine wave. In solutions of potassium chloride, they were found not to be reducible up to a potential of -1.8 volts. All of the latter amino acids except β-phenyl- β-alanine caused an appearance of a maximum in the cobaltous ion wave in unbuffered solutions. In buffered cobaltous solutions, β-phenyl- β-alanine caused a catalytic wave which is analogous to the cysteine wave, but not as sensitive, at a potential of -1.6 volts vs. the saturated calomel electrode. For reduction the substance must have a labile hydrogen which can be activated when the molecule is coordinated with cobaltous ions. The lability of the alpha hydrogen of β-phenyl- β-alanine was described earlier by Posner (9). The alpha hydrogen of β-phenyl- α-alanine is not as labile, therefore, this compound does not produce a catalytic wave.

Bridicka (10) studied the prenatrium wave and proposed that the presence of cystine groups in the protein was responsible for this wave also. The following facts supported his claim: (1) Proteins which do not contain cystine do not cause a prenatrium wave. (2) When cysteine is added to a simple buffer solution, a wave similar to the prenatrium wave occurs; however, since it appears at a more negative voltage it coalesces with the wave due to the buffer and becomes indistinct.

Jurka (11) later studied both the catalytic double wave and the prenatrium wave of protein simultaneously by electrolyzing buffered

cobalt solutions containing added amounts of proteins. The double wave appeared at -1.4 volts; whereas, the latter wave began at -1.5 volts. The prenatrium wave was found to be much larger than the catalytic double wave. She showed that the prenatrium wave was not obtained by earlier workers because they had used sensitivity settings of the instrument which could not record this wave. She concluded that all waves obtained were due to catalytic hydrogen deposition. The double wave was caused by protein sulfhydryl groups which are activated by cobalt, and the prenatrium wave was due to protein sulfhydryl groups non-activated by cobalt.

In 1952, Millar (12) (13), also studied the catalytic double wave and the prenatrium wave using five proteins, each of which has a different amount of "total potential RSH" groups (the sum of the cysteine and half-cystine residues). His results indicated that the sulfhydryl groups probably have the greatest influence in determining the magnitudes of the double wave; however, he was convinced that other protein groups are involved in the production of the prenatrium wave. Using the method of Stern and White (14) for the acetylation of the free amino groups and phenolic hydroxyl groups of insulin, he found a reduction of the prenatrium wave of about 80 per cent, while the catalytic double wave was only slightly affected. Addition of formaldehyde to protein buffer solutions was also shown to depress the prenatrium wave. Millar then repeated Bridicka's study of cysteine in buffered solutions and showed that the prenatrium wave produced by this compound was eliminated when the amino group was blocked by preparing the 2,4-dinitrophenyl derivative.

He concluded that the amino groups affect this wave by enhancing the tendency of the protein molecule to be adsorbed at the surface of the electrode, and are involved in electrode reactions which produce the wave according to the following reactions:

$$RNH_3^+ + e \longrightarrow RNH_2 + H$$

$$RNH_2 + H_3O^+ \longrightarrow RNH_3^+ + H_2O .$$

Millar also studied several smaller organic molecules containing amino groups and found that some of these produced a prenatrium wave although they did not contain sulfhydryl groups. Neither L-lysine monohydrochloride nor L-histidine monohydrochloride was found to have any effect when electrolyzed in potassium borate buffer at pH 8.

Several other workers have studied amino acids polarographically and found that no waves were produced in the absence of metallic ions which are readily complexed unless a "reducible" group like the disulfide group in cystine or the iodine in 3,5-diiodotyrosine is present. Simpson and Trail (15) found that both thyroxine and 3,5-diiodotyrosine produce polarographic waves and devised a method for the determination of thyroxine in the presence of the latter. Kolthoff and Barnum (16) found an anodic wave of cysteine at the dropping mercury electrode.

In 1941, Roberts (17) studied amino acids in cobalt salt solutions in an attempt to devise a polarographic method for detection of histidine in the presence of other basic amino acids. In the course of his work, he found that serine, tryptophan, and tyrosine are not reduced polarographically in lithium chloride solutions. Earlier, Pech (18) studied the reduction of some aliphatic amines and amino acids. He

found several of the amine salts to be reducible at the dropping mercury electrode. He reported also that glycine and asparagine are not reducible. No mention was made of the supporting electrolyte used.

Pleticha (19) studied fifteen amino acids in the presence of buffered and unbuffered solutions of Co^{++}, Ni^{++}, Fe^{++}, and Mn^{++}. The only waves he observed were waves due to the metal complexes and waves due to the presence of high concentration of H_3O^+ when acidic amino acids (aspartic or glutamic acid) were used.

The literature reviewed above includes all of the information available on the polarographic reduction of proteins and amino acids with alkali metal salts as supporting electrolytes. Although Pech used other supporting electrolytes, it is not stated in his report which one he used to obtain the results he reported for amino acids. Several supporting electrolytes have been used which have more negative reduction potentials than the alkali metal ions. Van Rysselberghe and McGee (20) and Von Stackelberg and Stracke (21) reported reduction potentials of -2.6 to -2.9 volts for quaternary salts of amines. These quaternary salts, because of their very negative reduction potentials, are useful as supporting electrolytes whenever the compound studied reduces at potentials more negative than those of the alkali metal ions.

In 1950, Childers and Gropp (22) found a group of mono- and dialkyl ammonium salts to be reducible at the dropping mercury electrode using tetraethyl ammonium bromide as the supporting electrolyte. They reported half-wave potentials ranging from -2.39 to -2.63 volts for the compounds studied. Amino acids can be considered primary amines in

which a hydrogen on the carbon adjacent to the functional group has been replaced by a carboxyl group. Thus, the work of Shikata and Tachi (23) becomes pertinent. In their studies of organic compounds, they found that the reduction potential of similar organic compounds follows closely the electronegativity of the groups combined with the reducible group.

Several workers have studied weak acids at the dropping mercury electrode (24) (25). Kolthoff and Lingane give a list of the half-wave potentials of a number of aliphatic and aromatic acids. On the basis of the Bronsted definition of acids and bases, all amino acids possess at least two functional groups which are potentially acidic. The dissociation constants of a number of amino acids have been reported by Schmidt (26). The dissociation constant of β-alanine was determined by Duggan and Schmidt (27). Tomes (24) developed an equation which shows that the half-wave potential of a weak acid should shift to more negative values with increasing acid concentrations.

In the work reported in this dissertation, several amino acids were studied with the polarograph using quaternary ammonium salts as supporting electrolytes. Analysis of polarographic curves was accomplished through the use of several equations developed by previous workers. The Ilkovic equation (28) allows us to determine diffusion coefficients from the wave heights. Diffusion coefficients of several amino acids have been determined by other methods (29) (30). The significance of the slopes of polarographic waves has been pointed out by Zimmerman and Gropp (31). Information concerning the mechanism of

reduction may be obtained from the first and second derivatives of a modified Nernst equation (32) using the value of the slope at the half-wave potential and the point of inflection of the polarographic wave.

CHAPTER I

EXPERIMENTAL AND RESULTS

Apparatus and Compounds

The Polarograph used in this investigation was a Sargent-Heyrovsky Model XII photographically recording type. The instrument has a sensitivity of 0.0087 microamperes per millimeter at a shunt ratio of 1. Instructions for installing, operating, and maintaining the polarograph are contained in a brochure available from the manufacturer.[1] The motor which drives the potentiometric bridge and camera is connected to a 110 volt A.C. power source. In order to take care of fluctuations in the power source, a constant-voltage transformer is employed.

A "Queen" Standard Potentiometer, Model E-3040-T, made by the Gray Instrument Company was used to measure the applied potentials. Three 22.5-volt dry-cell batteries connected in parallel served to supply the e.m.f. for the potentiometric circuit. These batteries were standardized against a Weston Standard Cell.

The dropping mercury electrode assembly used is made up of an electrolysis cell and a capillary connected to a mercury reservoir with high pressure, heavy-walled Neoprene tubing. The electrolysis cell used

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[1] Manual of Instructions for Sargent Model XII Polarograph, Chicago: E. H. Sargent and Co.

was the Erlenmeyer type originated by Heyrovsky in which a stationary pool of mercury on the bottom of the cell served as the internal anode. The height of the mercury reservoir was kept at 450 mm. for all of the experimental work. At this height, the capillary used was found to have a drop-time of 3.8 seconds. The mass of mercury flowing per second was 2.2 mg. when allowed to drop into distilled water under no applied potential. A diagram of the dropping mercury electrode assembly is shown on page 353 of Kolthoff and Lingane (2).

The applied potential as measured by the potentiometer depends upon the anode process, which, in turn, depends upon the supporting electrolyte used. In order to obtain significant values, the internal mercury pool anode potential must be measured against a standard reference electrode and the value subtracted from the measured potential. It is almost universal practice to express half-wave potentials with reference to the saturated calomel electrode. Throughout this investigation the anode pool was measured against a 0.1 N calomel electrode, constructed from a 125 ml. Erlenmeyer flask and equipped with an agar bridge. This 0.1 N calomel electrode was found to be +0.090 volts with reference to the saturated calomel electrode.

The mercury used in the dropping mercury electrode assembly and for the anode pool was cleaned by allowing it to drop in a fine stream through 3 molar nitric acid solution, 3 molar sulfuric acid solution, and distilled water. A final purification was obtained by distilling it under reduced pressure.

Three different supporting electrolytes were used in this

investigation. All of the compounds investigated were studied using tetraethylammonium bromide as the supporting electrolyte.[1] Since the half-wave potentials of the compounds studied are high, it was necessary to use a supporting electrolyte of this type because of its high reduction potential, -2.84 volts with reference to the saturated calomel electrode. The tetraethylammonium bromide was recrystallized several times from a mixture of n-propanol and anhydrous ethyl acetate until a polarogram of the supporting electrolyte solution showed it to be polarographically pure. Four to six recrystallizations were generally sufficient. When the half-wave potentials of some of the compounds studied were found to be sufficiently low, the other two supporting electrolytes, reagent grade lithium chloride[2] (-2.31 volts) and tetramethylammonium bromide[3] (-2.75 volts) were also used in order to compare the wave heights and half-wave potentials in different kinds of supporting electrolytes. The lithium chloride was used without further purification. The tetramethylammonium bromide was purified by recrystallizing four to six times from 80 per cent ethanol.

The compounds studied and their structural formulas are listed in Table 1. Glycine was purified by several recrystallizations from water-methanol mixtures according to the procedure given by Orten and Hill.[4] α-Alanine and β-phenylalanine were recrystallized from

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[1] Eastman Kodak Company.

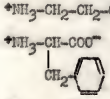
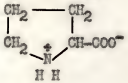
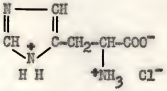
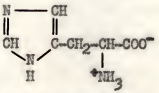
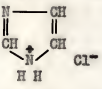
[2] J. T. Baker Chemical Company.

[3] The Matheson Company.

[4] "Organic Syntheses," Collective Vol. I, New York: John Wiley & Sons (1932).

TABLE 1

COMPOUNDS STUDIED

Compound	Structural Formula
Glycine[1]	$^{+}NH_3-CH_2-COO^{-}$
D,L- α-Alanine[2]	$^{+}NH_3-CH(CH_3)-COO^{-}$
β-Alanine[3]	$^{+}NH_3-CH_2-CH_2-COO^{-}$
D,L- β-Phenylalanine[2]	
L-Proline[2]	
L-Histidine monohydrochloride[2]	
D,L-Histidine[2]	
N-Acetyl glycine	$CH_3-CO-NH-CH_2-COOH$
Imidazole hydrochloride	

[1]Eastman Kodak Company.

[2]Nutritional Biochemicals Corporation.

[3]Purified student preparation.

water-ethanol mixtures. Proline, histidine hydrochloride, and histidine were found to be sufficiently pure to be used without recrystallization; that is, the polarographic waves were quite well-defined. The β-alanine was purified according to the method of Clarke and Behr.[1] Acetyl glycine was prepared according to the method of Herbst and Shemin.[1]

Imidazole hydrochloride was prepared from imidazole[2] in the following manner: Eight-tenths of a gram of imidazole was dissolved in 1.2 ml. of water. The solution was cooled in an ice-bath and concentrated hydrochloric acid solution was added dropwise until the pH was approximately 3. The solution was then evaporated until crystals began to appear. Several milliliters of absolute ethanol were added to dissolve the crystals and the solution was concentrated under vacuum on a steam bath until almost dry. The addition of alcohol and the process of concentrating were repeated several times to remove water and excess HCl. The resulting crystals were then dissolved in 1.5 ml. of hot absolute ethanol, 0.5 ml. of ether was added and the solution was cooled in an ice-bath until crystallization was complete. The crystals were then filtered, washed several times with ether and dried under vacuum. Since imidazole hydrochloride is very hygroscopic, it was kept in a vacuum dessicator whenever it was not being used.

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[1] "Organic Syntheses," Collective Vol. II, New York: John Wiley & Sons (1932).

[2] Eastman Kodak Company.

Procedures and Results

General Procedure for Obtaining Polarograms

The procedure given below is a general one that was selected to be followed for all of the compounds studied. An attempt was made to study all compounds in a concentration range of 1.0×10^{-4} to 4.0×10^{-3} molar. Certain limitations prevented this from being practicable for some of the compounds. As the concentration of a substance was increased, the polarographic waves tended to become irregular especially at their upper ends where voltages were close to the reduction potential of the supporting electrolyte. Another factor limiting the use of higher concentrations for some of the substances was the appearance of a maximum in the wave which made it difficult to evaluate the wave height, the half-wave potential and the slope. Thus, the highest concentration used for a particular substance was just below the concentration which would produce either too irregular a wave or too pronounced a maximum. The lower limit of concentration that could be used depended upon the minimum concentration which would produce a measurable polarographic wave. Preliminary investigations were carried out to determine the concentration range which would produce satisfactory waves for each of the compounds.

The preliminary investigations also indicated that the polarographic waves were more well-defined at lower temperatures than at 25° C., the standard temperature recommended for polarographic investigations.

The detailed procedure used was as follows: Ten milliliters of

freshly prepared 0.0500 molar tetraethylammonium bromide were placed in the electrolysis cell after which the cell was immersed in a water bath at 2-3° C. Nitrogen, which was saturated with water vapor by bubbling it through a flask of water, was then bubbled through the solution from 8 to 10 minutes to remove the dissolved oxygen. A polarogram was then recorded of this solution. Then 0.20 ml. of 0.0200 molar solution of the substance being investigated was added to the cell. Nitrogen was bubbled through the cell for two minutes to stir the solution. The dropping mercury electrode was introduced, the initial voltage determined with the potentiometer and the polarogram was recorded. When the wave due to the reduction of the supporting electrolyte began to appear, the recording was discontinued and the final voltage was determined. The dropping mercury electrode was then removed, the agar bridge from the 0.1 normal calomel electrode was immersed in the solution and the potential difference between the reference electrode and the anode was recorded. The camera setting of the polarograph was changed and another polarogram obtained at this concentration. Another 0.20 ml. of solution was added to the cell and the procedure described above was repeated. Polarograms were obtained in this manner after adding 0.20, 0.40, 0.70, 1.00, and 1.50 ml. of the solution. Thus, a series of polarograms were obtained for each compound studied.

Modified Procedures

Additional investigations were made on histidine hydrochloride and imidazole hydrochloride with some modifications of the general

procedure described above. Histidine hydrochloride was found to produce a wave which was at a sufficiently low potential to allow the use of other supporting electrolytes. This wave was also found to be well-defined at 25° C. Therefore, histidine hydrochloride was studied at 25° C. using lithium chloride, tetramethylammonium bromide, and tetraethylammonium bromide, respectively, as the supporting electrolytes. Otherwise, the procedure used was the same as previously described.

A second wave produced by histidine hydrochloride was measurable at a concentration of 10^{-5} molar. Therefore, the lower concentration limit of study for the second wave of this amino acid was extended to include several concentrations between 10^{-5} and 10^{-4} molar.

The potential at which the imidazole hydrochloride wave appeared was found to be low enough to use other supporting electrolytes. A series of polarograms were obtained for imidazole hydrochloride using lithium chloride as the supporting electrolyte and maintaining the temperature at 25° C. In contrast to the quaternary ammonium salts, lithium chloride solutions are very stable and stock solutions of it can be used.

Some preliminary work was undertaken to study the effect of changes in acidity on the polarographis waves of histidine hydrochloride. In general, the following procedure was used: Ten milliliters of 0.0500 molar lithium chloride solution were introduced into the cell and a wave recorded. Then 0.10 ml. of 0.02 molar hydrochloric acid solution was added and a wave recorded. Successive additions of small amounts of either 0.02 molar hydrochloric acid solution or 0.02 molar solution of

tetraethylammonium hydroxide[1] were introduced into the cell and polarograms obtained. Duplicate solutions were prepared and the pH of each solution was determined with a Beckman line-operated pH meter. The effect of pH on the waves of histidine hydrochloride solutions at two concentrations is shown in Figures 1 and 2.

Construction Methods of Analyzing Polarographic Waves

The polarographic wave obtained can be analyzed to obtain the following pertinent information: The wave height, often referred to as the diffusion current (i_d), the half-wave potential ($E_{\frac{1}{2}}$), the polarographic slope, and the polarographic point of inflection. Two typical polarographic waves of the types encountered in this investigation are shown in Figures 3 and 4. The waves of the type shown in Figure 3 are so close to the wave of the supporting electrolyte that no diffusion line is obtained. This figure is used to illustrate the construction method of evaluating diffusion currents, half-wave potentials, and polarographic slopes when no maximum is present. Figure 4 is used to illustrate how the diffusion currents were evaluated when the wave possesses a maximum.

Diffusion Current.---Line ab was drawn so that it was tangent to the residual current line. Line cd was drawn parallel to line ab through the point of inflection between the wave of the compound and the wave of the supporting electrolyte. This inflection point may be

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[1] Eastman Kodak Company.

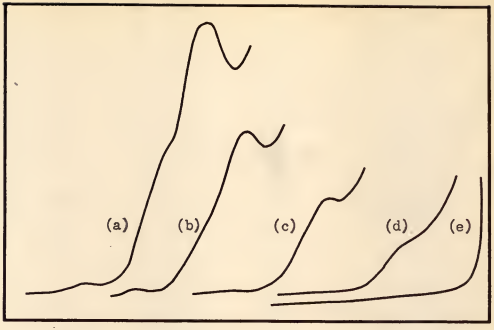

Figure 1.—Effect of pH changes on the first wave of a 4 X 10^{-4} molar histidine hydrochloride solution.
(a) pH 4.1 (b) pH 4.5 (c) pH 5.2 (d) pH 6.0
(e) supporting electrolyte.

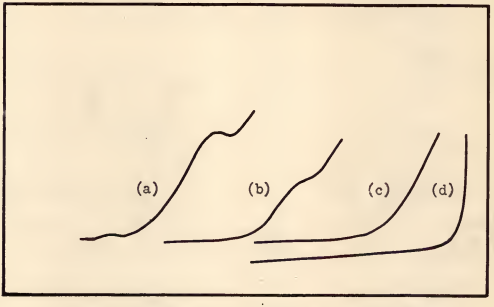

Figure 2.—Effect of pH changes on the first wave of a 3 X 10^{-4} molar histidine hydrochloride solution.
(a) pH 4.3 (b) pH 5.2 (c) pH 6.4 (d) supporting electrolyte.

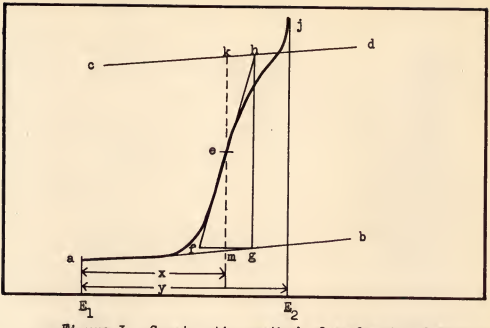

Figure 3.--Construction method of evaluating diffusion currents, half-wave potentials, and polarographic slopes.

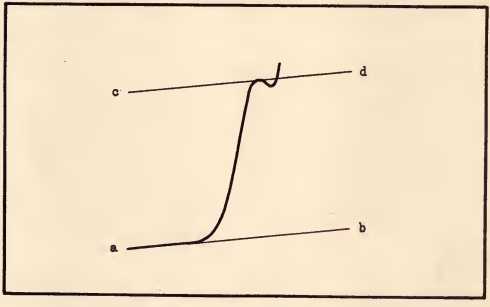

Figure 4.--Construction method of evaluating diffusion currents for a polarographic wave with a maximum.

obtained visually or by graphical methods. The height of the wave is the perpendicular distance, km measured in millimeters, between the two parallel lines ab and cd. The construction was the same in Figure 4 except that the line cd was drawn through the maximum. The diffusion current was then calculated in microamperes from the following equation:

$$i_d = km \times S \times 0.0087$$

where S is the shunt ratio at which the curve was run and 0.0087 is the sensitivity of the instrument in microamperes per millimeters. Figures 5 through 9 illustrate the effect of concentration on the diffusion current for the various compounds studied.

Half-Wave Potentials.--Perpendicular lines were dropped from points a and j in Figure 3. These points correspond to the initial and final voltages, E_1 and E_2 respectively. The distance y then corresponds to the total voltage span of the polarogram. The perpendicular line km was dropped at that point where ek was equal to em. Point e, therefore, corresponds to the half-wave potential and lines x and y can be related to the half-wave potential by the following equation:

$$E_{\frac{1}{2}} = E_1 - (E_2 - E_1)\frac{x}{y} - A$$

where A is the potential difference between the anode and the saturated calomel electrode. Half-wave potentials for the various compounds investigated are given in Table 2.

Polarographic Slope.--The line fh was drawn so that it was tangent to the curve at the half-wave. A perpendicular was dropped from point h until it hit the residual line ab at point g. The length of the voltage abscissa fg, when calculated in volts is reported as the slope

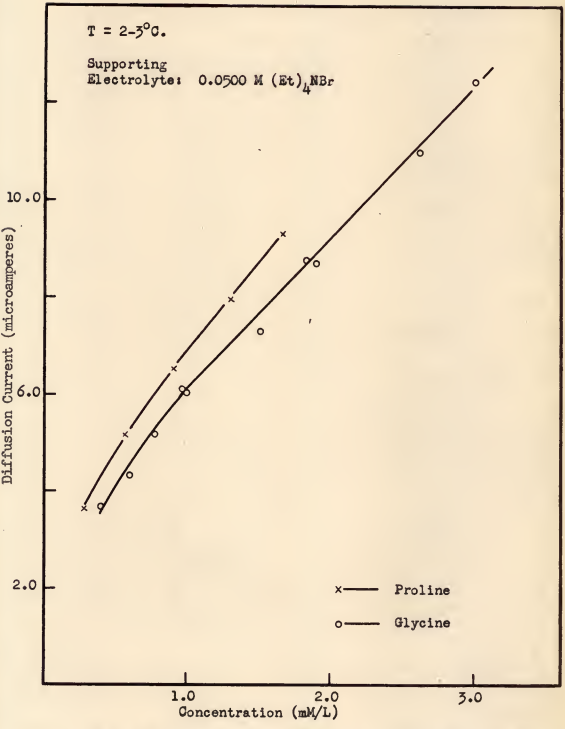

Figure 5.--Effect of concentration on diffusion current

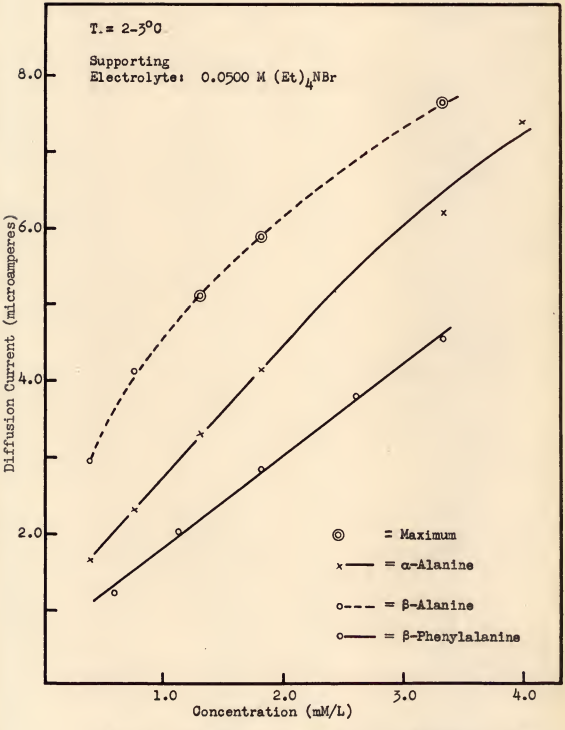

Figure 6.--Effect of concentration on diffusion current

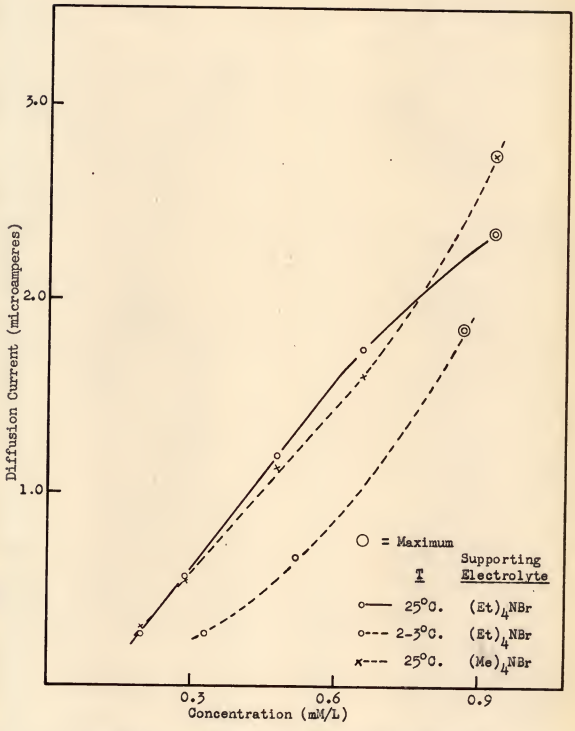

Figure 7.--Effect of concentration of histidine hydrochloride on diffusion current of first wave.

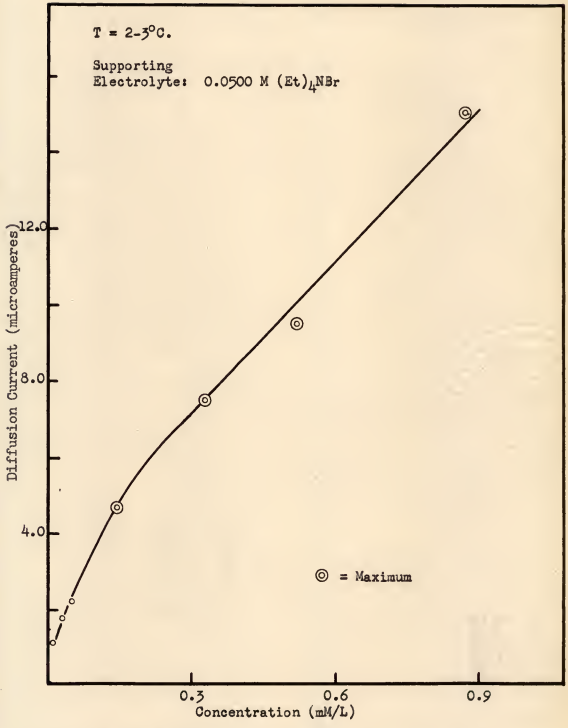

Figure 8.--Effect of concentration of histidine hydrochloride on diffusion of second wave.

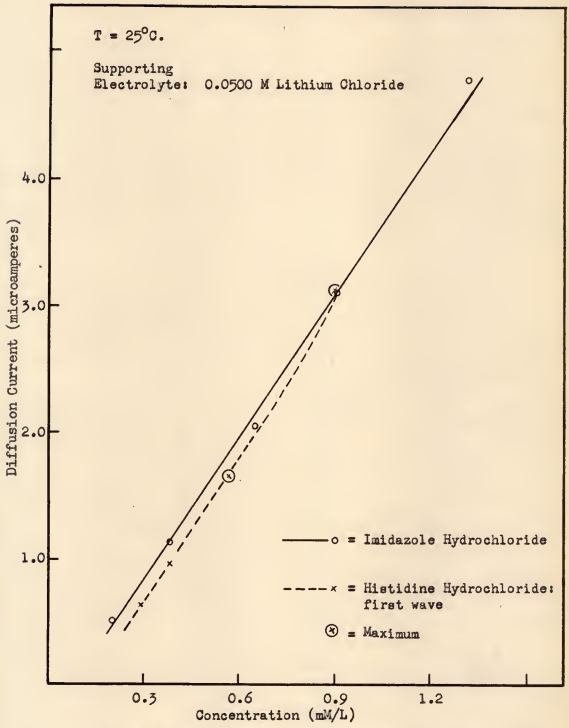

Figure 9.--Effect of concentration on diffusion current

TABLE 2

HALF-WAVE POTENTIALS WITH REFERENCE TO THE SATURATED CALOMEL ELECTRODE AT A TEMPERATURE OF 2-3° C. USING TETRAETHYLAMMONIUM BROMIDE AS THE SUPPORTING ELECTROLYTE

Compound	$-E_{\frac{1}{2}}$ (Volts)	Number of Determinations	Average Deviation
Glycine	2.614	11	0.005
α -Alanine	2.603	6	0.006
β -Alanine	2.601	5	0.010
β -Phenylalanine	2.536	5	0.003
Proline	2.635	9	0.010
Histidine hydrochloride	1.845	4	0.005
	1.699[a]	8	0.004
	1.791[b]	4	0.004
	1.764[c]	4	0.003
	2.460[d]	4	0.004
Imidazole hydrochloride	2.021	4	0.003
	1.832-1.882[e,a]	9	.....

[a]Supporting electrolyte: Lithium chloride. Temperature: 25° C.

[b]Temperature: 25° C.

[c]Supporting electrolyte: Tetramethylammonium bromide. Temperature: 25° C.

[d]Histidine hydrochloride produces a second polarographic wave.

[e]$-E_{\frac{1}{2}}$ varies with concentration.

at the half-wave. The values for the slopes for all compounds studied are listed in Table 3.

Polarographic Point of Inflection.—The point of inflection represents the spot at which the rate of change of the slope is zero. Therefore, a plot of the slope of the wave against the potential can be used to determine the point of inflection. Figure 10 is a graphically differentiated glycine curve. The potential at the point of inflection was found to be -2.69 volts. Using this value, the wave height at the point of inflection was obtained from the original glycine wave. The polarographic point of inflection is defined as the ratio of the wave height (i) at the point of inflection to the diffusion current (i_d). Points of inflection were determined for glycine waves only. The average of 6 values was found to be 0.74 with a mean deviation of 0.02.

Electrocapillary Properties

The Ilkovic equation, $i_d = 607n\ D^{1/2}\ C\ m^{2/3}\ t^{1/6}$, relates the diffusion current to the drop-time, t, and the mass of mercury flowing per second, m, at the half-wave potential. Since m and t vary with the applied potential, the values of m and t were determined at various potentials for solutions of several supporting electrolytes. The procedure for determining these values follows: Ten milliliters of 0.0500 molar supporting electrolyte solution were placed in an electrolysis cell. Nitrogen was bubbled through for several minutes and the dropping mercury electrode was immersed in the solution. The e.m.f. was increased until the desired potential was reached and the drop-time was determined.

TABLE 3

POLAROGRAPHIC SLOPES AT A TEMPERATURE OF 2-3° C. USING TETRAETHYLAMMONIUM BROMIDE AS THE SUPPORTING ELECTROLYTE

Compound	Slope (Volts)	Number of Determinations	Average Deviation
Glycine	0.36	6	0.02
α -Alanine	0.32	4	0.02
β -Alanine	0.26	4	0.01
β -Phenylalanine	0.32	7	0.02
Proline	0.34	5	0.02
Histidine hydrochloride	0.16	4	0.00
	0.18[a]	8	0.00
	0.20[b]	4	0.01
	0.21[c]	4	0.00
	0.38[d]	4	0.01
Imidazole hydrochloride	0.24	4	0.01
	0.24[a]	9	0.01

[a]Supporting electrolyte: Lithium chloride. Temperature: 25° C.

[b]Temperature: 25° C.

[c]Supporting electrolyte: Tetramethylammonium bromide. Temperature: 25° C.

[d]Histidine hydrochloride produces a second polarographic wave.

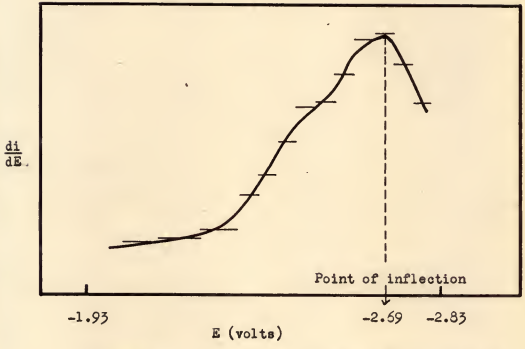

Figure 10.--Graphically differentiated glycine wave

With the potential set at this value, the dropping mercury electrode was transferred to a modified polarographic cell, which differs from the classical type in that it contains a funnel sealed into the bottom of the cell. This funnel serves to catch the mercury which drops from the electrode. The mercury was allowed to drop for a given period and m is calculated from the total mass of mercury and the time interval. Table 4 is a list of the electrocapillary properties of the particular capillary used in this investigation.

TABLE 4

OBSERVED RATE OF FLOW OF MERCURY FROM THE CAPILLARY AT DIFFERENT APPLIED POTENTIALS

Supporting Electrolyte 0.0500 M	-E (Volts vs. SCE)	Drop-Time (sec.)	Rate of Flow of Mercury (mg./sec.)
Lithium chloride[a]	1.83	2.22	2.17
	1.90	2.00	2.20
Tetraethylammonium[b] bromide	2.65	0.40	....
	2.75	0.15[c]	....

[a]The temperature was 25° C.

[b]The temperature was 2-3° C.

[c]Extrapolated value.

CHAPTER II

THEORY AND DISCUSSION

Whenever a substance produces a polarographic wave, it is possible to obtain a mechanism for the reduction process occurring at the dropping mercury cathode from the diffusion current, the polarographic slope, and the point of inflection of the wave.

According to Ilkovic, the theoretical equation for the diffusion current obtained with the dropping mercury electrode is,

$$i_d = 607\, n\, D^{1/2}\, C\, m^{2/3}\, t^{1/6},$$

where i_d is the average diffusion current, n is the number of Faradays of electricity required per mole of the substance reduced, D is the diffusion coefficient of the reducible substance in square centimeters per second, and C is the concentration in millimoles per liter. The term m is the rate of flow of mercury in milligrams per second and t is the drop-time in seconds at the applied potential. The chief application of diffusion coefficient data is for the evaluation of the n-value by use of the Ilkovic equation.

An examination of Figures 5 through 9 shows that in most cases the diffusion current is related linearly within limits to the concentration of the substances reduced. Using i_d/C values obtained from these curves, and the values for the diffusion coefficients reported by Mehl and Schmidt (30), the n-values were calculated for glycine, α-alanine, and proline using the above equation. Since i_d/C varied

with concentration for each of these amino acids, n was found to vary with concentration in each case. Therefore, the Ilkovic equation was not satisfactory for establishing the number of electrons involved in the electrode process. As reported by Kolthoff and Lingane (2), other workers have obtained similar results, when very rapid drop rates caused considerable stirring of the solution which disturbed the diffusion layer and produced an abnormally large current. In such cases, the linear relation between diffusion current and concentration may fail. Therefore, the Ilkovic equation was used to calculate diffusion coefficients at a concentration of 5×10^{-4} molar using a value of one for n, which will be shown later to be valid for one of the amino acids, glycine. Since the rate of flow of mercury was found to be independent of the applied potential, an average value of 2.20 mg./sec. was used for the value of m in the equation. When calculated results were compared with diffusion coefficients reported by other workers for some of the compounds, the calculated results were found to be extremely large; therefore, the results listed in Table 5 are reported as "calculated diffusion coefficients." The rate of flow of mercury at the potential of both the first wave of histidine hydrochloride and the imidazole hydrochloride wave was less than that at the higher potentials; therefore, stirring did not disturb the diffusion layer. Thus, the calculated diffusion coefficients for these two probably represent actual diffusion coefficients.

The geometric properties of polarographic waves can be used as a means of obtaining fundamental information regarding the nature of the polarographic process. The following mechanism for the primary electrode

TABLE 5

CALCULATED DIFFUSION COEFFICIENTS AT 2-3° C. WITH TETRAETHYLAMMONIUM BROMIDE SUPPORTING ELECTRODE

Compound[a]	i_d/C	$m^{2/3}t^{1/6}$	Calculated Diffusion Coefficient
Glycine	8.00	1.50	1.3×10^{-4}
α -Alanine	3.66	1.50	2.7×10^{-5}
β -Alanine	6.82	1.50	9.4×10^{-5}
β -Phenylalanine	2.24	1.62	7.3×10^{-6}
Proline	9.06	1.50	1.7×10^{-4}
Histidine hydrochloride	2.80[b]	2.02	5.7×10^{-6}
	18.3[c]	1.62	4.6×10^{-4}
Imidazole hydrochloride	3.16[b]	1.92	7.7×10^{-6}

[a]Concentration: 5×10^{-4} M.

[b]Lithium Chloride was supporting electrolyte. The temperature was 25° C.

[c]Second wave of histidine hydrochloride.

reaction, which is defined as the single process of the transfer of electrons from the dropping mercury cathode to the reducible substance, is proposed for the compounds investigated:

$$2\ \underset{\underset{R}{|}}{NH_2}\text{-}R' + 2\,e \longrightarrow 2\ \underset{\underset{R}{|}}{NH}\text{-}R' + H_2 . \qquad (1)$$

The proof of the mechanism is based on the assumption that the reaction is reversible and that there is established an adsorption equilibrium of the reduction product between the dropping mercury cathode and the solution. The succeeding equations which will be derived will enable the use of the polarographic slope and point of inflection in order to prove whether the proposed mechanism is correct.

For any given point on a polarographic wave, the applied potential across the polarographic cell is,

$$E_{app.} = E_a - E_c - iR \qquad (2)$$

where E_a is the anode potential, E_c is the potential at the dropping mercury electrode and iR is the potential drop due to the resistance of the polarographic cell. The presence of a high concentration of supporting electrolyte keeps E_a and iR constant. Thus, equation (2) can be written,

$$E_{app.} = E' - E_c . \qquad (3)$$

If reaction (1) occurs reversibly at the dropping mercury cathode then,

$$E_c = E_c^o - \frac{RT}{nF} \ln \frac{(A_p)^2(A_{H_2})}{(A_r)^2} \qquad (4)$$

where A_p and A_r are the activities of the product and the reactant,

respectively in solution at the surface of the mercury drop. Equations (3) and (4) can be combined to give,

$$E_{app.} = E'' + \frac{RT}{nF} \ln \frac{(A_p)^2(A_{H_2})}{(A_r)^2} . \qquad (5)$$

The use of a high concentration of supporting electrolyte maintains a constant ionic strength. Therefore, the activity coefficients are constant and the equation now becomes,

$$E_{app.} = E''' + \frac{RT}{nF} \ln \frac{(C_p)^2(C_{H_2})}{(C_r)^2} . \qquad (6)$$

The rate of diffusion of reactant to the dropping mercury electrode is proportional to the concentration gradient between the substance in the bulk of the solution and the depleted surface. The current at any point on the wave is,

$$i = k_1(C_r^o - C_r) \qquad (7)$$

where C_r^o is the concentration of the reducible substance in the bulk of the solution. When the diffusion current is attained, C_r is negligibly small and

$$i_d = k_1 C_r^o . \qquad (8)$$

By combining equations (7) and (8), the following expression is established for C_r at any point of the wave;

$$C_r = \frac{i_d - i}{k_1} . \qquad (9)$$

In the case of the reaction products, they will be considered as formed and present for the major part in an adsorbed state at the surface of the mercury drop. The product in the solution near the

the mercury drop is in equilibrium with the product adsorbed at the surface of the mercury according to the Freundlich equation,

$$C_p = K C_{ps}^{z_1} \tag{10}$$

where C_{ps} is the concentration of product adsorbed at the surface of the mercury and z_1 is a parameter with a value greater than one. The concentration of the product adsorbed on the surface of mercury is proportional to the current,

$$C_{ps} = K_2 i . \tag{11}$$

Therefore,

$$C_p = K(k_2 i)^{z_1} = K_1 i^{z_1} . \tag{12}$$

It can also be shown that,

$$C_{H_2} = K_2 i^{z_2} . \tag{13}$$

By replacing C_r, C_p, and C_{H_2} with the above values in equation (6), it becomes, after rearranging,

$$E_{app.} = E'''' + \frac{RT}{nF} \ln \frac{i^Z}{(i_d - i)^2} \tag{14}$$

where $Z = 2z_1 + z_2$. (15)

Then substituting $u = i/i_d$, equation (14) now becomes,

$$E_{app.} = E^x + \frac{RT}{nF} \ln \frac{u^Z}{(1-u)^2} . \tag{16}$$

This is the equation for the applied potential at any point of a polarographic wave for a given concentration.

If equation (16) is differentiated with respect to u, the slope of the wave is obtained. At the half-wave, $u = 1/2$ and the equation

for the slope at the half-wave becomes,

$$\frac{dE}{du} = \frac{RT}{F}(Z + 2) \qquad (17)$$

The second derivative with respect to u, when set equal to zero is the point of inflection of the wave.

$$\frac{d^2E}{du^2} = \frac{RT}{2F}\left[\frac{2}{(1-u)^2} - \frac{Z}{u^2}\right] = 0 \qquad (18)$$

Therefore,

$$\frac{2}{(1-u)^2} = \frac{Z}{u^2} . \qquad (19)$$

The geometric properties of the glycine wave were used to test the validity of the postulated mechanism. Glycine was selected because it has the simplest structure of the amino acids and the waves produced were generally quite well-defined. Using the measured slope of glycine of 0.36 volts, a value of 13.2 for Z was calculated from equation (17). When this value of Z was substituted into equation (19), a value of 0.72 was calculated for the inflection point. The measured point of inflection, from the graphical differentiation of the wave, is 0.74. This agreement between theoretical and experimental values establishes the postulated mechanism (1). Because of the similarity in structure of all of the compounds studied, it is probable that this mechanism also holds for the others.

The reduction, therefore, depends upon the release of a proton by the reducible group. Further evidence that the reduction process depends upon a proton release was obtained by studying acetylglycine,

a derivative of glycine in which the assumed functional group ($^{+}NH_3$-) is blocked. No polarographic wave was produced by acteylglycine at or near -2.6 volts, the potential at which the glycine wave occurs.

The compounds studied, with the dissociation constants of the groups which are thought to be the proton donors in the reduction reaction are listed in Table 6. Since the mechanism depends upon a proton release, it was expected that the acid strength of the functional group would have a large effect on the ease of reduction; that is, the weaker the acid, the more negative the half-wave potential. In Figure 11, the half-wave potentials listed in Table 2 are plotted against the pK_a values of the functional group involved in the reduction. Examination of the graph shows that half-wave potentials become more negative with decreasing acid strength. A quantitative measure of the effect of pK_a on half-wave potentials was obtained by using the method of least squares to obtain the following empirical equation relating half-wave potentials and pK_a values:

$$E_{1/2} = -0.187\ pK_a - 0.718.$$

Since histidine hydrochloride produced two polarographic waves, the wave at -1.845 volts was attributed to the imidazole grouping (pK_a = 6.10), while the wave at -2.460 volts was attributed to the $^{+}NH_3$- group (pK_a = 9.18). When a base is added to histidine hydrochloride, the first acid-base reaction which occurs results in the loss of a proton by the imidazole grouping in the compound. This should result in the disappearance of the wave which occurs at the less negative potential. It can be seen from Figure 2, that the wave height of the

TABLE 6

DISSOCIATION CONSTANTS REPORTED IN THE LITERATURE FOR THE COMPOUNDS STUDIED

Compound	K_a	pK_a	Determining Group
Glycine	1.66×10^{-10}	9.78	$^+NH_3-$
α -Alanine	1.35×10^{-10}	9.87	$^+NH_3-$
β -Alanine	5.13×10^{-11}	10.29	$^+NH_3-$
β -Phenylalanine	5.75×10^{-10}	9.24	$^+NH_3-$
Proline	2.52×10^{-11}	10.60	$^+NH_2=$
Histidine Hydrochloride	7.95×10^{-7}	6.10	$^+NH_2=$
	6.61×10^{-10}	9.18	$^+NH_3-$
Imidazole Hydrochloride	8.30×10^{-8}	7.08	$^+NH_2=$

first wave of a 3×10^{-4} molar histidine hydrochloride solution decreases with increasing pH until it disappears at a pH of 6.4. At this pH, the concentration of the acid form was calculated to be 1×10^{-4} molar, a concentration too low to yield a polarographic wave.

In order to investigate further the imidazole grouping of histidine hydrochloride, a study of imidazole hydrochloride was undertaken because of its similarity in structure to the former compound. The acid group of imidazole hydrochloride is weaker than the corresponding group in histidine hydrochloride. For this reason, polarograms of solutions of imidazole hydrochloride were expected to show a wave at

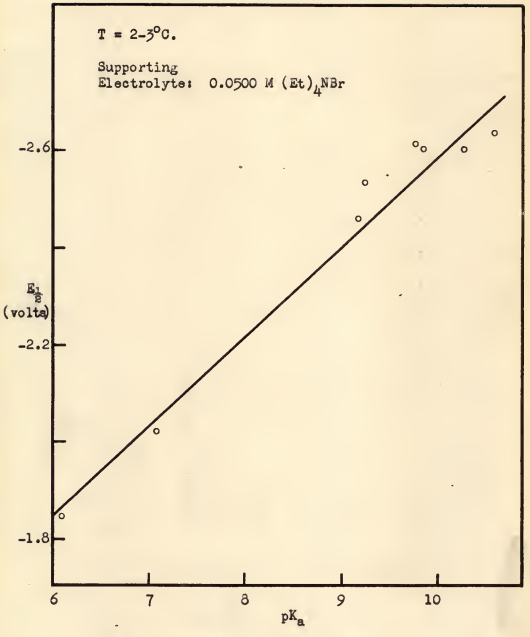

Figure 11.--Effect of acid strength on half-wave potentials of the compounds studied.

potentials more negative than the first wave of histidine hydrochloride. The experimental results show that such is the case; that is, the wave produced by imidazole is 0.15 volts more negative than that of histidine hydrochloride.

CHAPTER III

SUMMARY

The half-wave potentials of glycine, α-alanine, β-alanine, β-phenylalanine, proline, and histidine hydrochloride, all of which were found to produce polarographic waves, were determined using tetraethylammonium bromide as the supporting electrolyte. The effect of concentration on diffusion current was also determined for each amino acid.

The following mechanism was established for the reduction process of glycine using the geometric properties of its wave:

$$2\ {}^{+}NH_3\text{-}CH_2\text{-}COO^{-} + 2\ e \longrightarrow 2\ NH_2\text{-}CH_2\text{-}COO^{-} + H_2\ .$$

It is suggested that a similar mechanism holds for the other compounds studied.

A relation was found between the acid strengths of the reducible groups and the half-wave potentials of the compounds. This relation was in agreement with the proposed mechanism which involves a proton release by the reducible group. The following empirical equation, which relates acid strength of the reducible group and half-wave potentials of the compounds, was obtained:

$$E_{\frac{1}{2}} = -0.187\ pK_a - 0.718.$$

Imidazole hydrochloride was studied because of its similarity in structure to histidine hydrochloride. Values for this compound of the half-wave potential and the diffusion current were determined.

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BIOGRAPHICAL NOTES

Victor Hyman Dayan was born in St. Petersburg, Florida, on April 26, 1927. He attended St. Petersburg Junior College in 1943 and 1944. He entered the University of Florida in 1945 and received his Bachelor of Science degree in Chemistry with honors in September, 1947. Mr. Dayan was employed as an analytical chemist by the Plant Food Division of Swift & Co., Bartow, Florida, in 1947. He returned to the University of Florida in 1949 to pursue his graduate studies. He held positions in the Department of Chemistry of graduate assistant and teaching assistant.

He is a member of Gamma Sigma Epsilon Chemical Fraternity and Phi Sigma Biological Society.

This dissertation was prepared under the direction of the chairman of the candidate's supervisory committee and has been approved by all members of the committee. It was submitted to the Dean of the College of Arts and Sciences and to the Graduate Council and was approved as partial fulfillment of the requirements for the degree of Doctor of Philosophy.

June 7, 1954

C. J. Byers
Dean, College of Arts and Sciences

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